Evaluation of a method for isolation and purification of sporocysts of the cephalopod coccidian parasite *Aggregata* Frenzel, 1885 (Apicomplexa: Aggregatidae)

Evaluación de un método para el aislamiento y purificación de los esporoquistes de *Aggregata* Frenzel, 1885 (Apicomplexa: Agregatidae), coccidios parásitos de cefalópodos

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Recibido el 25-VII-1999. Aceptado el 10-IX-1999

ABSTRACT

A modified simple method of isolation and purification of the sporocysts of the parasitic apicomplexan genus *Aggregata* Frenzel, 1885, which parasitize the digestive tract of cephalopods, is described. This method involves Sucrose and Percoll density gradient and takes about two hours. The average amount of material was 0. 5 million of purified sporocysts per gram of heavily-parasitized host tissue. It may become a routine procedure for subsequent ultrastructural and molecular studies on cephalopod coccidians.

RESUMEN

En este trabajo se describe un método modificado, sencillo, para el aislamiento y la purificación de esporoquistes del parásito apicomplexa del género *Aggregata* Frenzel, 1885, que parasita el tracto digestivo de cefalópodos. Este método incluye la realización de gradientes de Sacarosa y Percoll y se completa en dos horas aproximadamente. Se consigue una cantidad media de 0, 5 millones de esporoquistes por g. de tejido hospedador fuertemente parasitado. Esta técnica puede considerarse un proceso rutinario para posteriores estudios ultraestructurales y moleculares en coccidios de cefalópodos.

KEY WORDS: *Aggregata*, coccidians, sporocysts, parasites, cephalopods, purification.
PALABRAS CLAVE: *Aggregata*, coccidios, esporoquistes, parásitos, cefalópodos, purificación.

INTRODUCTION

In the last few years procedures for detecting and monitoring the pathology and parasitic diseases of molluscs has been introduced on a wide scale in the NW Spanish fishing and farming industry as an integral part of a general disease assessment policy. Review of current research stresses the importance of epizootiological information on the spatio-temporal distribution of the commonest diseases/parasites in many wild and farmed molluscan populations. Re-

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cently, PASCUAL, GESTAL, ESTÉVEZ, RODRÍGUEZ, SOTO, ABOLLO AND ARIAS (1995) have demonstrated the role of commercially-exploited cephalopods as intermediate or final hosts in the life cycles of at least ten systematic groups of parasites. Even though several not yet described *Aggregata* species are known to occur (some of them with a low prevalence) in several ommastrefid and octopod hosts (HOCHBERG, 1990), the prevalence of infection for the eimeriorin coccidian *Aggregata* Frenzel, 1885 was close to 100% in both the common octopuses and the European cuttlefishes (PASCUAL *ET AL.*, 1995).

The genus *Aggregata* is an intracellular coccidian parasite with a two-host life cycle. Sexual stages gamogony and sporogony occur in the digestive tract of cephalopods, the definitive host, and asexual stages merogony, in the digestive tract of crustaceans, the intermediate hosts.

Although the pathogenicity of *Aggregata* in cephalopods is still inconclusive (FORD, 1992), this coccidian parasite is believed to be an important factor in reducing host condition in massive infections (DOBELL, 1925). POYNTON, REIMSCHUESSEL AND STOSKOPF (1992), by examining parasitized sections of the caecum of *Octopus dofleini* and *O. bimaculoides* from the East North Pacific Ocean, noted that invaded host cells undergo considerable nuclear and cytoplasmic hypertrophy and the nuclei became displaced to one side. The submucosa was sometimes ulcerated and fibrosis and necrosis were occasionally observed. In heavy infections, the host tissue was almost completely replaced by parasites. The same histopathological diagnosis was noted in parasitized *Octopus vulgaris* and *Sepia officinalis* from the NE Atlantic Ocean (GESTAL, ABOLLO, ARIAS, GUERRA AND PASCUAL, 1997).

The first and major obstacle in assessing the effects of parasites in wild and reared cephalopod populations is the ability to quantify such effects (PASCUAL, GESTAL AND ABOLLO, 1997). While the process of analysing histopathological changes related to parasitic infections is typically qualitative and strongly coupled to the experience of the observer, quantitative descriptors of host-parasite populations are needed. Among them, infection intensity (i. e., the number of individuals in an infrapopulation) is a very convenient descriptive statistic because hosts are discrete and natural sampling units (BUSH, LAFFERTY, LOTZ AND SHOSTAK, 1997). In this way, knowledge of parasite- host dynamics especially the spatial distribution of parasites within host are of considerable interest because of their consequences for regulation of parasite and host populations (CROFTON, 1971; ANDERSON AND MAY, 1978; ANDERSON GORDON, 1982; KURIS AND LAFFERTY, 1992), and their importance in developing an effective parasite monitoring programme (ANDERSON AND MAY, 1985).

Secondly, different species or subspecies, even sub-populations from batches of oocysts of a parasitic apicomplexan genera (e. g., *Plasmodium* and *Toxoplasma*) contain multi-functional virulence determinants on their surfaces that allow the parasite to establish successfully the infectious cycle (COX, 1993). Although each of the protozoan parasites has its own peculiarities and hurdles, it appears likely that in the near future our understanding of virulence in different *Aggregata* species (i. e., field-type cysts with different size and shape) could be possible if an effective, simple and rapid method of isolation and purification was available.

Thirdly, the classification and nomenclature within the coccidian genus *Aggregata* has, historically, been controversial which brings into question the validity of previous and future epidemiological studies. Despite the reasons for the taxonomic and systematic controversy, the problem can only be resolved by agreement on an appropriate species concept and on operational procedures for implementing that concept. The development and maintenance of isolated, purified, viable coccidian stock collections would then allow taxonomic comparisons between previously described and newly isolated coccidia, permit

preservation of potentially useful biological control agents, and conserve basic genetic material. The ability to examine and compare previously described species that have not been examined structurally or with molecular techniques is fundamental to developing a phylogenetically based system of classification.

The solution to the above problems is an important area for coccidiosis research in the near future. To this triple-aim, we evaluate a simple method as a tool for improving the isolation and purification of the sporocysts of *Aggregata* from infected cephalopod tissues. The technique may help to enhance studies on the effects of coccidiosis and to stage infected hosts for further research on the development of cephalopod coccidia through ultrastructural and molecular studies.

MATERIAL AND METHODS

Host sampling and processing: Common octopus *Octopus vulgaris* Cuvier 1797 and common cuttlefish *Sepia officinalis* Linnaeus 1758 infected with the coccidia *Aggregata octopiana* and *A. eberthi*, respectively (PASCUAL *ET AL.*, 1995; ESTÉVEZ, PASCUAL, GESTAL, SOTO, RODRÍGUEZ AND ARIAS, 1996) were collected in artisanal gears by local fishermen from the Ría of Vigo (NW Spain), and immediately transported alive to the laboratory. During necropsy, small pieces of spiral caecum (the preferred site of infection; ESTÉVEZ *ET AL.*, 1996) were excised and cleaned in distilled water to remove residual food. To confirm the existence of sporogonic stages, squash preparations of fresh infected material was examined by ligth microscopy. Coccidian oocysts contain sporocysts which look like spherical, delicate balls 11-15 (m in diameter in *A. octopiana* and 8-9 (m in diameter in *A. eberthi* with 6-12 and 3 sporozoites (the infective form), respectively. Only those pieces of host tissue which are seen macroscopicaly highly parasitized with oocysts of *Aggregata* were taken into experiments.

Description of the method: isolation and purification of Aggregata sporocysts using Sucrose and Percoll density gradient centrifugation (Fig. 1): Host tissue extracts (5-10 g. depending on the size of the host), were prepared by homogenisation in an electric tissue grinder (IKA-UltraTurrax T-25) containing 75 ml distilled water with a 18 G dispersing tool at 9500 1/min of speed dispersion (5 min, and then with a 10 G dispersing tool at 8000 1/min of speed dispersion x 10 min. This process provided an available dispersion from 10 to 50 μm. The recovered sporocysts were then observed separated from the remaining host debris and also from most of the bacteria. Tissue homogenates were filtered through two-layered nylon gauze sieves of 100, 60, 40, and 20 (m of pore diameter (Millipore Corporation, Bedford, USA) to remove larger pieces of host tissue. Sporocysts and small pieces of host tissue were obtained after filtering. The filtrate can be stored at 4°C before use by adding sodium azide (Sigma Chemical Co., St Louis, USA). The filtrate was centrifuged in a Beckman GS-15R fixed-angle rotor centrifuge at 1000g (5 min to pellet the sporocysts; the supernatants with only cellular residual components was discarded. The spores were washed by centrifugation 2-3 times with distilled water until the supernatant was clear, and then passed to saline solution 0, 9%. Sporocysts were further purified by density gradient centrifugation for the elimination of all host tissue obtaining a sample of pure sporocysts. Sucrose (Sigma Chemical Co., St Louis, USA) was diluted with saline solution 0.9% to concentrations of 80%, 60%, 40%, and 20%, respectively. When making a discontinuous gradient tube, 10 ml of 80% Sucrose suspension was first added into a 50 ml test assay tube followed successively by equal volumes of 60%, 40%, and 20% Sucrose concentrations. Finally, 5 ml of the sporocyst suspension was layered onto the top of the gradient tube. After centrifugation at 170 g (5 min. for *A. octopiana*, and 250 g (5 min. for *A. eberthi*, three bands were formed, appearing as milky interfaces

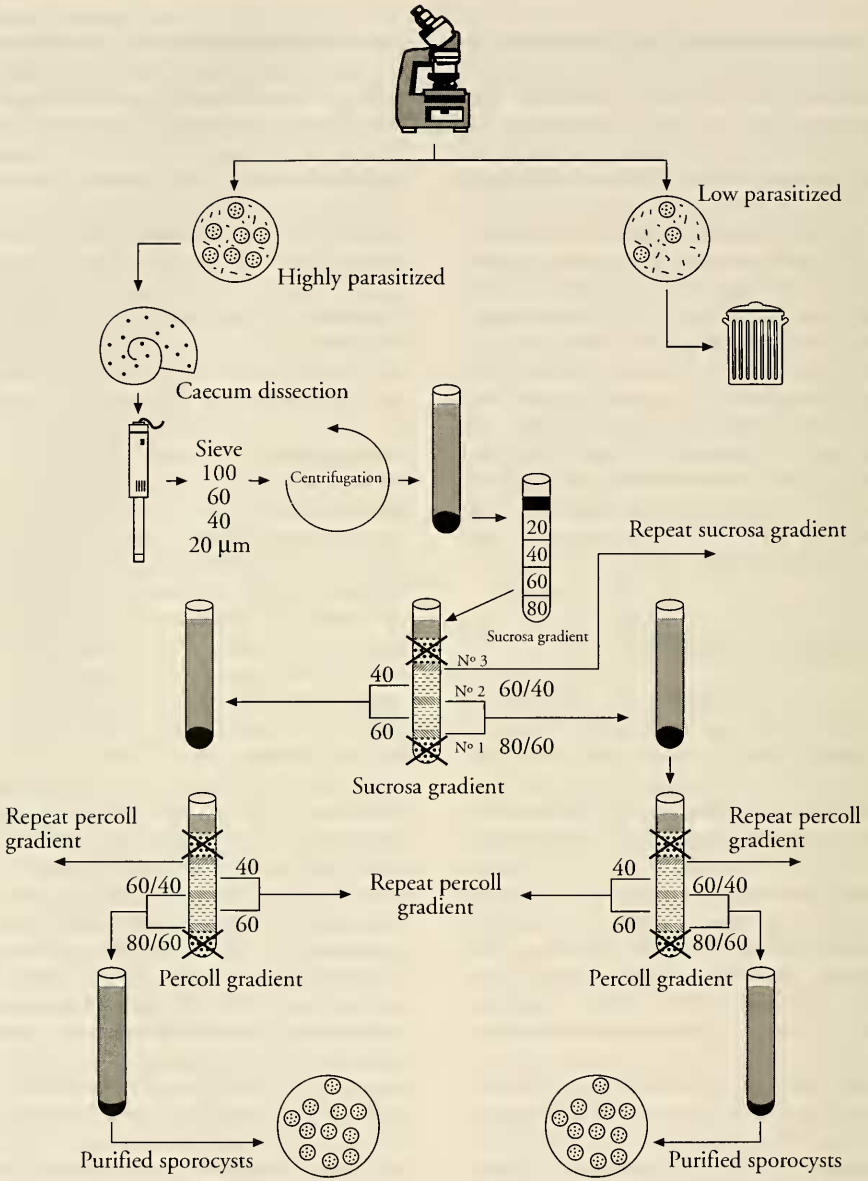

Figure 1. Diagram of *Aggregata* sporocyst purification process.
Figura 1. Diagrama del proceso de purificación de esporoquistes de Aggregata

between each consecutive layer. Bands in interface 80%-60% (N° 1), and interface 60%-40% (N° 2) were recovered using a syringe, transferred together to a separate tube and diluted in saline solution 0.9%. The interface 40%-20% (N° 3) trapped a great amount of sporocysts but with a lot of host tissue debris, so it was necessary to repeat the above procedure with this interface separately. The sample of the 40% and 60% suspension layers were recovered together too

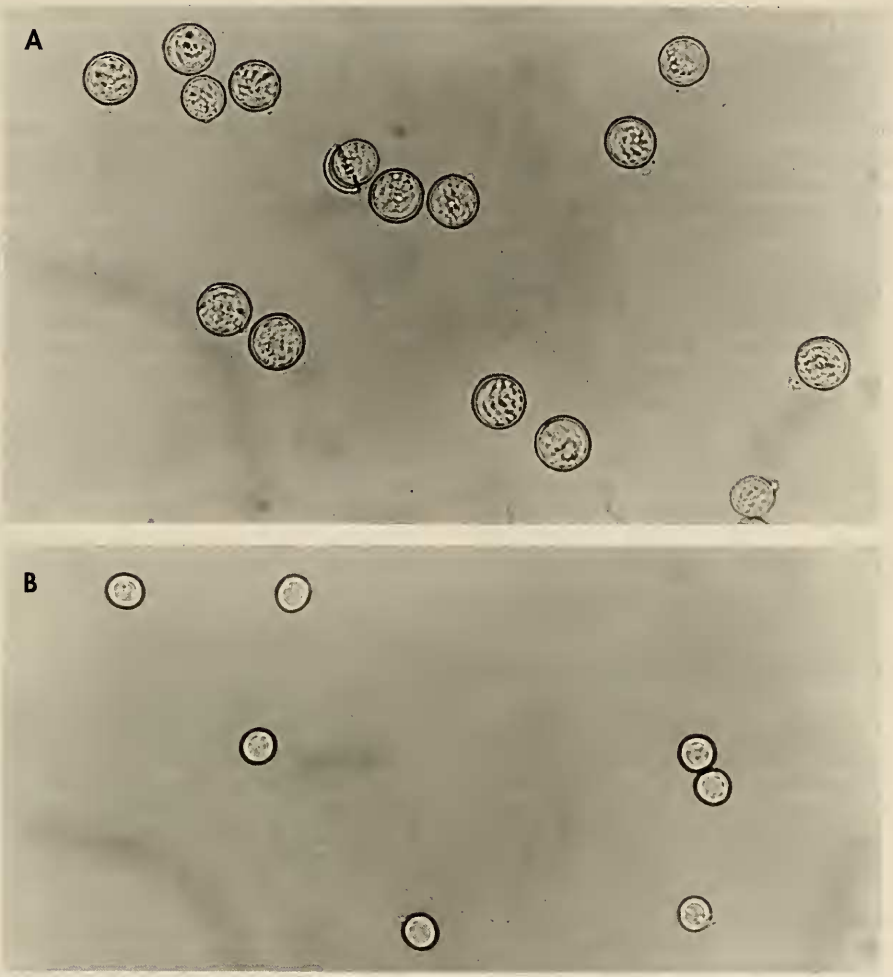

Figure 2. Fresh preparations of purified sporocysts. A: sporocysts of *Aggregata octopiana*; B: Sporocysts of *Aggregata eberthi.*
Figura 2. Preparaciones frescas de esporocitos purificados. A: esporoquistes de Aggregata octopiana; *B: esporoquistes de* Aggregata eberthi.

in another separate tube (N° 4) and diluted in saline solution 0.9%. The layer of 20% usually trapped nothing except for small host tissue debris, and the 80% only very large host tissue debris, sometimes some sporocysts, but in a very low percentage, so those capes normally are discarded.

Percoll ((Pharmacia, Uppsala, Sweden) discontinuous gradient (80%, 60%, 40%, and 20% in saline solution), was used after a new centrifugation at 1000g (5 min. on samples N°1-2 and 4 separately. The pellet of the centrifuged samples were resuspended each in 1ml saline solution, added on the Percoll discontinuous gradient of layers of 2ml each in a assay tube of 10 ml and centrifuged at 100 g (5 min. for *A. octopiana* or 170 g (5 min. for *A. eberthi.* Similar bands and layers to those of the Sucrose gradient were formed. The 20% and 80%

layers, like in the Sucrose gradient, trapped different sizes of host tissue debris, so those layers are discarded. The band formed in the interface 80%-60%, and those of the interface 60%-40%, containing purified sporocysts (Figure 1), were transferred together to a separate tube, centrifuged at 1000 g (5 min, resuspended in 1 ml of saline solution and stored at 4°C with the addition of sodium azide or frozen in distilled water or the specific buffer depending on which technique was used. The interface 40%-20% trapped sporocysts but not completely purified, so it was necessary to repeat twice the same procedure of Percoll gradient with this interface separately. The sample of the 40% and 60% layers trapped sporocysts with a large degree of purification, but it is advisable to repeat once the Percoll gradient to obtain completely purified sporocysts.

EVALUATION AND DISCUSSION

With the offered technique we obtained different layers and bands formed in interfaces after gradient centrifugation on Sucrose and Percoll, containing large number of isolated, purified sporocysts of *A. octopiana* and *A. eberthi*. Neither faecal debris nor bacteria nor other contaminants could be observed at the end of the purification procedure (Figure 2), and the recovered sporocysts preserved their morphological characteristics when light microscopy techniques was carried out. This modified method described in this paper has been specifically developed for purifying large numbers of *Aggregata* sporocysts from infected cephalopod tissues. It takes about two hours and the average proportion of suitable material was of 0.5 million of purified sporocysts per gram of heavily-parasitized host tissue. The usefulness of this modified method can be extrapolated to the purification procedure of the sporocysts of other *Aggregata* species from the digestive tract of other host species.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Angel Guerra of Instituto de Investigaciones Marinas (CSIC) for critical comments on drafts of this manuscript. This work was partially supported by the Spanish Grant CICYTMAR- 95, 1919- C05.

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